Full Methods

Yeast strains and plasmids. Standard cloning and yeast techniques were used for construction, transformation and integration of plasmids[1-3]. HA-tagged versions of *HAC1* with either its own 3'UTR or that of *ACT1* or *PGK1* were integrated as a genomic copy, replacing endogeneous *HAC1*. The splicing reporter (SpR) construct was generated by replacing positions 1 to 648 of the *HAC1* coding sequence in exon1 with the GFP ORF. In the Δ3'BE mutants, positions 176-182 and 212-218 of *HAC1's* 3'UTR were deleted. The 3'BE stem that was placed between the stop codon and the *ACT1* 3'UTR of the SpR comprised positions 155-187 and 207-236 of *HAC1's* 3'UTR. The mRNA visualization constructs were created by inserting into the pRS426 vector[4] the sequences of *PGK1*, *HAC1*, or — a non-fluorescent GFP-R96A mutant of — the splicing reporter ending at position 280 of *HAC1*'s 3'UTR, followed by 16 tandem repeats of the U1A binding sequence and the *PGK1* terminator, derived from pPS2037 (a kind gift from Roy Parker), and a polyA signal. A copy of the U1A RNA binding domain fused to GFP was integrated into the genome from plasmid pRP1187 (a kind gift from Roy Parker). Surprisingly, the key to the low noise in the imaging lies in the curious fact that in pRP1187 the U1A-GFP ORF is inserted backwards, so that its expression is driven by a cryptic, uncharacterized promoter element within the (reverse) *PGK1* transcription terminator. The low levels of U1A-GFP expression derived from this construct prove ideal for mRNA imaging. By PCR, a previously described[5] 5' stem loop structure was introduced 26 nucleotides upstream of *PGK1*'s start codon, and nucleotides 108-280 of the *HAC1* 3'UTR, comprising the entire stem, were inserted after the *PGK1* stop codon, where indicated. A monomeric (A206R), yeast codon adapted version of GFP, derived from pKT127[6], or mCherry was placed into Ire1 between residues I571 and G572, and the FKBP derived Fv2E domain (Ariad) between R112 and Y449, replacing the core ER stress sensing domain[7]. Ire1 interface mutants are: if1 (T226W/F247A), if2 (W426A), and if1/2 (T226W/F247A/W426A)[7]. Ire1 variants in all assays were expressed at near endogenous levels from centromeric pRS315.

RNA and protein analysis. RNA preparation, electrophoresis, labeling of probes for Northern blot analysis and quantitation of splicing efficiencies were performed as

described[8]. Protein extraction, electrophoresis, transfer to nitrocellulose for immunoblot analysis with α-HA antibody were performed as described[9].

Microscopy. All samples were taken from yeast cells that were kept in early log phase for at least 24 h in synthetic media containing excess amounts of adenine and tryptophan before imaging. Light microscopy was done with a Yokogawa CSU-22 spinning disc confocal on a Nikon TE2000 microscope. GFP was excited with the 488nm Ar-ion laser line and cherry with the 568 nm Ar-Kr laser line. Images were recorded with a 100x/1.4 NA Plan Apo objective on a Cascade II EMCCD. The sample magnification at the camera was 60 nm/pixel. The microscope was controlled with μManager and ImageJ. Images were selected for analysis and for display in figures to contain no saturated pixels (in case of the RNA imaging) and a signal significantly above background (in case of Ire1-mCherry imaging). We excluded images of cells with strong vacuolar auto-fluorescence. Images were processed in ImageJ and Adobe Photoshop such that the linear range of the signal was comparable between images.

Quantitative analysis of Ire1 foci and co-localization of mRNA in foci. Images of Ire1-mCherry and U1A-GFP decorated *HAC1*^{U1A}, SpR^{U1A} and *PGK1*^{U1A} mRNAs and variants thereof were analyzed using a customized MatLab script to determine the fraction of Ire1-mCherry in foci and to score the recruitment of U1A-GFP decorated mRNA in Ire1 foci. The annotated MatLab script is available (Supplementary File). In brief, the mean pixel intensity of a background area (~20% of section area) was defined in an intracellular area excluding ER. Ire1 was defined as all signal exceeding the mean background by 1.1–fold. Under non-stress conditions, we never observed this signal to exceed a 1.5-fold background threshold. We thus defined the fraction of Ire1 in foci as the ratio of Ire1-Cherry fluorescence intensity above threshold divided by the total Ire1-Cherry fluorescence intensity. The threshold was empirically defined such that under non-stress conditions no signal was scored as “foci”. Similarly, RNA foci were defined as pixels exceeding twofold the mean intensity in the RNA channel. A “co-localization index” was then defined as the integrated intensity of the pixels within the RNA foci that had pixels in common with Ire1 foci divided by the total RNA intensity and expressed in arbitrary units in a range of 0 to 100. Per condition, the percentage of Ire1-mCherry in foci and the co-localization index for the mRNA recruited to the foci was determined for

3-9 individual cells. Values and the standard error of the mean are given in histograms in Figures 2-4. Since, in contrast to the covalently fluorescently tagged Ire1, we do not know what fraction of the fluorescent reporter U1A-GFP in cells is bound to mRNAs containing U1A binding sites, background subtraction for U1A-GFP was arbitrary. Therefore, we report co-localization by this "co-localization index" rather than by an absolute % co-localization measure. The co-localization index robustly scores the differences in mRNA recruitment we observed in the fluorescent micrographs.

Methods References

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